



## The role of freshwater copepods in the environmental risk assessment of caffeine and propranolol mixtures in the surface water bodies of Spain



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### HIGHLIGHTS

- Propranolol is toxic to *Diacyclops crassicaudis crassicaudis*, while caffeine is non-toxic.
- The CA model predicts the toxicity of propranolol and caffeine mixture for this species.
- ERA is presented for caffeine and propranolol in the freshwater bodies of Spain.
- Caffeine poses an environmental risk to all the freshwater ecosystems of this study.
- Propranolol poses an environmental risk to some freshwater ecosystems of Spain.

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### ABSTRACT

In this study we aimed at assessing: (i) the environmental risk posed by mixtures of caffeine and propranolol to the freshwater ecosystems of Spain; (ii) the sensitivity of freshwater copepod species to the two compounds; (iii) if the toxicity of caffeine and propranolol to freshwater copepods contributes to the environmental risk posed by the two compounds in the freshwater bodies of Spain. The environmental risk was computed as the ratio of MECs (i.e. the measured environmental concentrations) to PNECs (i.e. the respective predicted no-effect concentrations). The effects of caffeine and propranolol on the freshwater cyclopoid *Diacyclops crassicaudis crassicaudis* were tested both individually and in binary mixtures. Propranolol posed an environmental risk in some but not in all the surface water ecosystems of Spain investigated in this study, while caffeine posed an environmental risk to all the investigated freshwater bodies, both as single compound and in the mixture with propranolol. Propranolol was the most toxic compound to *D. crassicaudis crassicaudis*, while caffeine was non-toxic to this species. The CA model predicted the toxicity of the propranolol and caffeine mixture for this species. *D. crassicaudis crassicaudis* was much less sensitive than several other aquatic species to both compounds. The sensitivity of *D. crassicaudis crassicaudis* does not increase the environmental risk posed by the two compounds in the freshwater bodies of Spain, however, further testing is recommended since the effect of toxicants on freshwater copepods can be more pronounced under multiple stressors and temperature increasing due to climate change.

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## 1. Introduction

During the last three decades, pharmaceutical and personal care products (PPCPs) have received increasing attention in freshwater monitoring programs worldwide (Ebele et al., 2017; Kristofco and Brooks, 2017; Saari et al., 2017; Zhang et al., 2018). PPCPs may enter freshwater bodies directly in agricultural areas due to run off and landfill leaching, or indirectly via aquaculture discharges, industrial and urban wastewater treatments plants (WWTPs) and septic tanks (Larsson et al., 2007; Larsson, 2014). Despite the improvement of WWTP efficiency in the last years, a wide range of PPCPs is detected at concentrations in the  $\text{ng L}^{-1}$  to  $\mu\text{g L}^{-1}$  range in both surface water and groundwater bodies worldwide (Fatta-Kassinios et al., 2011; Ebele et al., 2017; Mutiyar et al., 2018). PPCPs detected in freshwater bodies (surface-, groundwater and groundwater associated ecosystems) encompass pesticides, antibiotics, analgesics, anti-inflammatories, lipid regulator agents,  $\beta$ -blockers, antiepileptics, contraceptives, steroids and hormones (Hernando et al., 2006; Meffe and de Bustamante, 2014; Ebele et al., 2017).

Caffeine is the most commonly consumed stimulant by humans with a daily global consumption exceeding 460 tons (Buerge et al., 2003). It is also used as a cardiac, cerebral, respiratory stimulant and diuretic (Buerge et al., 2003). An unknown portion of caffeine makes its way through wastewater-treatment plants globally, so that it is among the most frequently detected PPCPs in EU freshwater bodies (de Sousa et al., 2018) where it was found with concentrations up to  $50,000 \text{ ng L}^{-1}$  (Česen et al., 2019). Propranolol hydrochloride is a non-selective  $\beta$ -adrenergic receptor blocking agent, prescribed for the treatment of angina, hypertension, migraine and anxiety (Mehvar and Brocks, 2001). Although propranolol is considered to be readily biodegradable, it has been detected in high concentrations in surface water of several EU countries such as France, Germany, UK and Southern Spain (Hilton and Thomas, 2003; Ferrari et al., 2004). PPCPs occur in mixtures almost everywhere (Ebele et al., 2017). Mixtures of propranolol and caffeine were detected in EU freshwater bodies, such as in Spain (Camacho-Muñoz et al., 2010; Fernández et al., 2010) and Turkey (Ayman and Işık, 2015).

The occurrence of PPCPs in EU may be an impediment to the achievement, or maintenance, of the good ecological status of EU freshwater bodies *sensu* Water Framework Directive 2000/60/EC. To obtain a comprehensive overview of the effects of PPCPs on EU freshwater ecosystem quality, the EU regulations have encouraged environmental risk assessment (ERA) studies concerning these compounds (EMA, 2006, 2018). The rationale of ERA is the computation of the ratio of the measured environmental concentrations (MECs) of PPCPs to the Predicted No-Effect Concentrations (PNECs) i.e. to the concentrations below which unacceptable adverse effects on freshwater biota are not expected to occur. PNEC values are computed from toxicity data of the most representative freshwater taxa encompassing at least three different trophic levels, namely primary producers (algae and macrophytes), primary (invertebrates, mainly *Daphnia*) and secondary (vertebrates, mainly fish) consumers (EMA, 2006, 2018). However, data concerning the sensitivity of freshwater biota to PPCPs are still scarce (Deblonde and Hartemann, 2013). The available studies indicate that the persistence and continuous release of PPCPs in the environment expose freshwater organisms to these compounds throughout their entire life-cycle (Fabbri, 2015; Ebele et al., 2017). The risks of PPCP mixtures might substantially exceed the risk posed by each individual mixture component; nevertheless the effect of PPCP mixtures on freshwater species has been very poorly investigated up to now (Kortenkamp et al., 2009).

Among the four freshwater free-living copepod orders

(Calanoida, Cyclopoida, Harpacticoida and Gelyelloida), the Cyclopoida, and mainly the species belonging to the family Cyclopidae, are widely distributed in freshwater ecosystems (Galassi et al., 2009). Cyclopoids are omnivorous and feed on organic detritus, microbes, protists, fungi and algae (Suárez-Morales, 2015). They can also behave as predators exerting strong selection pressures on cladocerans and rotifers in planktonic environments (Suárez-Morales, 2015). Surface water cyclopoid species have a relatively short life-cycle and are easy to rear in the laboratory (Di Marzio et al., 2009, 2013; 2018; Di Lorenzo et al., 2015a; Cifoni et al., 2017). Their use in ecotoxicological studies has been recommended (Kulkarni et al., 2013; Cifoni et al., 2017). However, very little is known about their sensitivity to PPCPs (Di Lorenzo et al., 2014 and references therein).

In this study we aimed at assessing: (i) the environmental risk posed by mixtures of caffeine and propranolol to surface water ecosystems in Spain; (ii) the sensitivity of freshwater copepod species to the two compounds; (iii) if the toxicity of caffeine and propranolol to freshwater copepods contributes to the environmental risk posed by the two compounds in the freshwater bodies of Spain. To this end, we investigated the acute effects of caffeine and propranolol, both individually and in a binary mixture, on the juvenile stages of the freshwater cyclopoid *Diacyclops crassicaudis crassicaudis*, a cosmopolitan species frequently planktonic in ponds and also found in benthic layers of streams, rivers, springs and saturated alluvial and karstic aquifers (Reid, 1992). Sometimes erroneously considered stygobite (i.e. obligate groundwater-dweller), this species prefers surface water bodies (Reid, 1992). Along with the tests with the copepod species, we carried out a set of acute bioassays with the freshwater standard species *Daphnia magna*, the test invertebrate species most commonly used for ecotoxicological analyses in surface freshwaters. We selected caffeine and propranolol based on their high consumption and detection frequency in EU aquatic environments (Ebele et al., 2017). Since propranolol is able to antagonize the effect of caffeine in humans and rats (Babey et al., 1994; Humayun et al., 1997), an antagonistic effect in the mixture of the two compounds was expected for *D. magna* and *D. crassicaudis crassicaudis*.

## 2. Materials and methods

### 2.1. Chemicals and dilution water

The toxicity tests were carried out with analytical reagent-grade caffeine (CAF; CAS 58-08-2) and propranolol hydrochloride (PRO; CAS 318-98-9), both purchased from Sigma-Aldrich (Steinheim, Germany). Chemical properties of the two compounds are shown in Table 1. Dilution water was prepared in the laboratory with MILLIPORE® MILLI-Q® deionised water remineralized with reagent grade chemicals to obtain the following chemico-physical composition:  $\text{pH} = 8.26$ ,  $\text{EC} = 265 \mu\text{S cm}^{-1}$ , hardness =  $90.71 \text{ mg L}^{-1}$ ,  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O} = 60 \text{ mg L}^{-1}$ ,  $\text{MgSO}_4 = 60 \text{ mg L}^{-1}$ ,  $\text{NaHCO}_3 = 96 \text{ mg L}^{-1}$ ,  $\text{KCl} = 4 \text{ mg L}^{-1}$  (Cifoni et al., 2017). Four stock solutions (two per each compound) were prepared dissolving each compound in the dilution water. Per each trial, 4 or 5 concentrations (Table 2) for each pharmaceutical compound were prepared by diluting the appropriate volumes of stock solutions. The experimental concentrations were chosen based on preliminary range-finding tests.

### 2.2. Test organisms

Specimens of *D. crassicaudis crassicaudis* were sampled from a borehole drilled in the shallow porous aquifer of Jarama, 40 km southwest of Madrid ( $40^{\circ} 23' 32.52''\text{N}$ ,  $3^{\circ} 30' 17.02''\text{W}$ , 578 m a.s.l.) in October 2017. Measurements of the chemico-physical

**Table 1**

Investigated compounds with their analytical parameters and physico-chemical properties. MM: molecular mass ( $\text{g mol}^{-1}$ ); S: solubility at  $25^\circ\text{C}$  ( $\text{mg L}^{-1}$ ); Log  $K_{\text{ow}}$ : octanol-water partition coefficient.

Compounds	Elemental composition	MM	S	Log $K_{\text{ow}}$	Therapeutic use
Caffeine	$\text{C}_8\text{H}_{10}\text{N}_4\text{O}_2$	194.19	21600	-0.07	Central nervous system stimulant
Propranolol hydrochloride	$\text{C}_{16}\text{H}_{21}\text{NO}_2 \cdot \text{HCl}$	295.80	50000	-0.45	Non-cardioselective $\beta$ -adrenergic antagonist

**Table 2**

Measured concentrations in  $\text{mg L}^{-1}$  of caffeine (CAF) and propranolol hydrochloride (PROP) in the acute tests with *Daphnia magna* (Dma) and *Diacyclops crassicaudis crassicaudis* (Dcr). The concentrations were measured at the end of the tests. The toxic units are indicated in brackets.

Species	Concentration	Single compound bioassays		Mixture bioassays		
		CAF	PROP	CAF	PROP	$\Sigma$ Mix
Dma	C1	54	0.47	24.4(0.07)	0.25(0.05)	24.65(0.12)
Dma	C2	162	1.32	33.8(0.09)	0.26(0.05)	34.06(0.14)
Dma	C3	243	4.12	48.1(0.13)	0.32(0.06)	48.42(0.19)
Dma	C4	486	12.7	98.4(0.27)	0.50(0.10)	98.9(0.37)
Dma	C5	729		185.5(0.51)	0.60(0.12)	186.1(0.63)
Dma	C6					
Species	Concentration	CAF	PROP	CAF	PROP	$\Sigma$ Mix
Dcr	C1	207	4.9	54.7(0.01)	1.44(0.04)	56.14(0.06)
Dcr	C2	306	9.7	71.4(0.02)	2.06(0.06)	73.46(0.08)
Dcr	C3	408	14.6	235(0.06)	2.89(0.08)	237.89(0.15)
Dcr	C4	493	19.9	658(0.18)	6.73(0.19)	664.73(0.38)
Dcr	C5		20.3	2838(0.78)	15.16(0.43)	2853.16(1.21)

parameters were performed *in situ* using a portable multimeter probe for electrical conductivity (Crimson CM 35), dissolved oxygen (Crimson OXI 45P), pH (PH 25) and temperature (Crimson CM 35). In addition, 1 L of bore water was collected for further laboratory analyses of the following parameters (OECD, 2004): non-purgeable organic carbon (NPOC), total organic carbon (TOC), total carbon (TC), inorganic carbon (IC),  $\text{DBO}_{\text{C}_5, 20}$  (respirometric method with incubator bottles from Oxitop), Chemical Oxygen Demand (COD), hardness and alkalinity. The major ions were analysed by ion chromatography. Total suspended solids (TSS) were analysed by filtering the water through an AP40 Millipore filter. Data are shown in the Supplementary File (BW).

Cyclopoid specimens were collected from the bottom and the water column of the borehole using a phreatobiological net with a mesh of 63  $\mu\text{m}$  (Di Lorenzo et al., 2014; Di Marzio et al., 2018). After collection, the specimens were transported to the lab with the bore water within 1 h. In the lab, the copepods were sorted using a glass micropipette under a stereomicroscope at 12  $\times$  magnification. Specimens of *D. crassicaudis crassicaudis* were maintained at the conditions of the habitat from which this species was collected, that is in the original bore water, in 500 mL vessels (max 100 individuals per vessel), at the temperature of  $15 \pm 0.2^\circ\text{C}$  (the mean annual temperature of the bore water) in a laboratory thermostatic cabinet (Velp Scientifica™ FOC 120E Cooled Incubator) in permanent darkness. Bore water was renewed every other weeks. Specimens were kept in the bore water to allow them to feed on the microbes of their native habitat (Di Lorenzo et al., 2014, 2015a,b, 2016; Di Marzio et al., 2009, 2013, 2018).

*D. magna* was obtained from the continuous laboratory stock of the National Institute for Agricultural Research and Experimentation (INIF, Madrid). The specimens were cultured in the lab in the Elendt M4 standard water (OECD, 2004), at  $18 \pm 0.2^\circ\text{C}$  (the lowest temperature allowed for the test; OECD, 2004) and natural light conditions and fed with *Chlorella* to ensure  $1.7 \times 10$  cell  $\text{mL}^{-1}$  in the culture medium. The algal cells of *Chlorella* were cultured separately in OECD TG 201 medium (OECD, 2011), collected by centrifugation, and resuspended in the standard water. Algae were

regularly harvested while still in the exponential growth phase (5–7 days old) and inoculated in fresh medium.

### 2.3. Experimental design

The single compound test with *D. crassicaudis crassicaudis* was run according the protocols of Di Marzio et al. (2009, 2018) and Di Lorenzo et al. (2014) that are briefly described hereafter. The toxicity tests were performed with juvenile stages (C3-C5 copepodids). To ensure the number of copepodids for the tests, 8 ovigerous females per each compound were separated from the original stock culture three days after collection and maintained in a 200 mL-glass vessel under the same rearing condition of the culture. After 12–15 days (average development time of C3-C5 copepodid stages at  $15^\circ\text{C}$ ) the copepodids were picked up from the culture and randomly loaded in the test vials. The tests started within 3 weeks after collection. Before starting the tests, the copepodids were acclimated for 3 days in 200 mL of the dilution water at the temperature of  $15 \pm 0.2^\circ\text{C}$ , being the temperature in the collection site in the  $13^\circ\text{C}$ – $17^\circ\text{C}$  range. During acclimation the individuals were deprived of food in order to allow the gut to empty completely (Di Marzio et al., 2009, 2018; Di Lorenzo et al., 2014). Gut emptiness was confirmed visually under stereomicroscope at 80  $\times$  magnification before the tests. Faeces were removed, and only actively swimming copepodids were selected for the tests. The assays were carried out in sterile plastic plates 5 cm in diameter (Costar®). Two mL of each test solution were provided for each individual. Five specimens were placed into each test vessels after filling the plates with 10 mL of the test solutions. Hence, twenty individuals, divided into four groups of five each, were used per each test concentration and for the controls. Controls were set up with the dilution water. All the tests were run for 96 h at  $15 \pm 0.2^\circ\text{C}$  in permanent darkness. No food was offered during the trials. The test vessels were not aerated during the test. Every 24 h, each well was observed under a stereomicroscope (Olympus ZSX-7) for the presence of dead animals (specimens showing no movement after gentle stimulation by a sorting needle for 15 s. Spasms were not

counted as movements). Whenever necessary, the test solutions were added to each well every 24 h to maintain the final volume of 10 mL in each vessel. We drew a level mark at 10 mL on the vials and added the solution with a glass pipette. Exposure solution was never renewed during the trials to avoid stress due to manipulation.

The single compound test with *D. magna* was based on the standard OECD protocol (OECD, 2004). Ovigerous females of *D. magna* were separated from the original stock culture and maintained in 200 mL glass vessels filled with the dilution water used for the test solutions. Less than 24 h-old neonates were used for the trials. Daphnids were picked up from the culture and loaded in the test vials. The controls were set up with the dilution water used for the stock culture. Four replicates, each containing five daphnids, were assayed per each concentration so that a total of 20 daphnids were tested per each concentration (a total of 100–120 daphnids were used per each compound). Tests were run at  $18 \pm 0.2^\circ\text{C}$  under 16-h light and 8-h dark cycle. The test vessels were not aerated during the test. The daphnids were not fed during the test. The test duration was 48 h (OECD, 2004). Each test vessel was checked for immobilised daphnids at 24 h and 48 h after the beginning of the test. Whenever necessary, the test solutions were added to each well every 24 h to maintain the final volume. Exposure solution was never renewed during the trials.

Binary mixture experiments were carried out at  $15^\circ\text{C}$  for *D. crassicaudis crassicaudis* and at  $18^\circ\text{C}$  for *D. magna*. The adopted mixture ray design (van Gestel and Hensbergen, 1997) was based on toxic unit scaling as showed in Table 2. The equivalent toxic units (TU), defined as the ratio of the actual concentration (C) of a substance to the concentration that is needed to cause a certain effect, ECx (Backhaus et al., 2004), are indicated in brackets in Table 2.

All the tests fulfilled the validation requirements established in the OECD (2004) guidelines, that is: i) in the controls not more than 10% of the specimens were found dead/immobilised (*D. crassicaudis crassicaudis*: 0% for caffeine, 3% for propranolol and 0% for the mixture; *D. magna*: 0% in all the trials); ii) the dissolved oxygen concentration at the end of the tests was always  $>3\text{ mg L}^{-1}$  in control and test vessels. Dissolved oxygen was measured with the Crimson OXI 45P probe.

#### 2.4. Chemical analyses

For each compound a stability study was performed during four weeks to depict the respective potential degradation by exposing them to light and distinct temperatures as following: 1) darkness,  $-22^\circ\text{C}$ ; 2) darkness,  $-4^\circ\text{C}$ , and 3) light,  $20^\circ\text{C}$ . The study was performed to select the best conditions for storing the stock solutions before the tests and the working and test concentrations after the tests. Each concentration was tested three times.

At the end of the acute tests, the effective exposure concentrations were analysed, taking water samples from the surplus of the working solutions and from the test vials. The samples were analysed by high-pressure liquid chromatography system (HPLC) coupled to a UV–Vis detector (Agilent Technologies 1260, Palo Alto, CA, USA). The chromatographic separation was carried out at  $25^\circ\text{C}$  on a Phenomenex Luna C<sub>18</sub> analytical column (150 mm × 3 mm, 3 μm particle size). Gradient elution was performed with 10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> or NH<sub>4</sub>Ac/HAc in MilliQ water (pH = 6) as mobile phase A and acetonitrile as mobile phase B. Calibration graphs were run with the standard water used for the acute test.

#### 2.5. Statistical analyses

EC50 (*D. magna*) and LC50 (*D. crassicaudis crassicaudis*) values and the corresponding 95% confidence limits for the number of

immobilised and dead organisms in the acute toxicity tests for each compound were determined by nonlinear regression analysis, fitting a logistic equation to the data using the technique of least squares. The library “drc” and the software R vs. 3.5.0.0 (R Development Core Team, 2013) were used.

Multiple analysis regression was used to fit the observed binary mixture toxicity data to caffeine and propranolol concentrations. The full model was:

$$\text{ECx} = \beta_0 + \beta_1[\text{caff}] + \beta_2[\text{prop}] + \beta_3[\text{caff}] \times [\text{prop}] + \epsilon \quad (1)$$

where  $\beta$  values represent the regression coefficients and  $\epsilon$  is the normal error. To evaluate the concentration addition (CA) approach, the concentrations of the two compounds in the mixture expected to result in 50% of effect were calculated by solving the multiple regression for one of the two compounds having the highest slope value while holding the concentrations of the other constant (Gopalapillai and Hale, 2015). These combinations of concentrations were used to calculate the “sum of toxic units” for each mixture test case, as follows:

$$\sum \text{TU} = \sum_{i=1}^n \frac{\text{Ci}}{\text{EC50i}} \quad (2)$$

where Ci denotes the concentration of the ith toxicant in the mixture causing 50% of mortality and EC50i is the concentration of the ith toxicant causing same effect in a single chemical exposure. When  $\sum \text{TU} = 1$ , CA occurs. Deviations from unity can occur, as when the sum is more than unity (indicating that the mixture effect is less-than additive or antagonism) or when the sum is less than unity (indicating more-than additive or synergism).

#### 2.6. Environmental risk assessment

The ERA for each individual compound was based on the computation of the Risk Quotient (RQ) as following:

$$RQ = \frac{\text{MEC}}{\text{PNEC}} \quad (3)$$

where MEC is the measured environmental concentration of the compound and PNEC is its predicted no-effect concentration. Following an approach used for the development of water quality standards (Calamari and Vighi, 1992; Vighi et al., 2003), the RQ for the mixture of the two compounds (RQ<sub>mix</sub>) was calculated as:

$$\text{RQ}_{\text{mix}} = \sum_{i=1}^n \frac{\text{MEC}_i}{\text{PNEC}_i} = \sum_{i=1}^n \text{RQ}_{\text{ind}} \quad (4)$$

where n is the total number of PPCPs analysed in each sampling site (in this study n = 2). RQ and RQ<sub>mix</sub> exceeding 1 indicates the need for either a more refined risk assessment by including additional representative species (EMA, 2006; Di Lorenzo et al., 2018), and/or the implementation of risk mitigation measures. RQ > 1 or RQ<sub>mix</sub> > 1 imply an unacceptable environmental risk (EMA, 2006; Hernando et al., 2006; Di Lorenzo et al., 2018).

In this study, MEC values were the highest concentrations measured in the surface water bodies of Spain to account for a worst-case evaluation. MECs were obtained from the available literature using ISI Web of Knowledge SM (© 2010 Thomson Reuters) with the following keywords: “[pharmaceutical compound name] and Spain and river or surfacewater or water or WWTP or waste\*”. The results of the online search are shown in Table 3a,b. In this Table we indicated the highest MECs along with the mean MECs whenever available.

**Table 3a**

Risk quotients (RQs) computed as the ratio of the highest measured environmental concentration (MEC) in  $\mu\text{g L}^{-1}$  values of caffeine (CAF), propranolol (PRO) and the mixture of the two compounds to the respective PNEC values in  $\mu\text{g L}^{-1}$  following Approach 1. Mean MECs are indicated in brackets. \*: mean computed from min and max values. \*\*: median. Surface water bodies at risk are indicated in bold in the column "Risk".

PPCP	PNEC	Surface water body	MEC	Reference	RQ	Risk
CAF	0.001	Guadiamar River	2.05 (0.025)	Garrido et al. (2016)	2050	<b>At risk</b>
CAF		Guadalquivir River	0.23 (0.092)	Robles-Molina et al. (2014)	230	<b>At risk</b>
CAF		Guadalquivir River	0.74 (0.39*)	Fernández-Gómez et al. (2013)	740	<b>At risk</b>
CAF		Guadiamar River	2.72 (0.68)	Camacho-Muñoz et al. (2010)	2720	<b>At risk</b>
CAF		Guadiamar River	2.50 (1.37)	Camacho-Muñoz et al. (2010)	2500	<b>At risk</b>
CAF		Jarama River	2.14 (1.57)	Valcárcel et al. (2011)	2140	<b>At risk</b>
CAF		Manzanares River	13.17 (12.92)	Valcárcel et al. (2011)	13170	<b>At risk</b>
CAF		Guadarrama River	4.72 (2.84)	Valcárcel et al. (2011)	4720	<b>At risk</b>
CAF		Heneses River	0.67	Valcárcel et al. (2011)	670	<b>At risk</b>
CAF		Tagus River	2.08	Valcárcel et al. (2011)	2080	<b>At risk</b>
CAF		Guadiamar River	1.10 (0.63)	Camacho-Muñoz et al. (2010)	1100	<b>At risk</b>
CAF		Henares-Jarama-Tajo Rivers	0.41(0.059**)	Fernández et al. (2010)	410	<b>At risk</b>
PRO						
PRO	3.18	Guadiamar River	0.0083	Garrido et al. (2016)	0.3	No risk
PRO		Guadiamar River	0.64 (0.26)	Camacho-Muñoz et al. (2010)	2.0	<b>At risk</b>
PRO		Guadiamar River	0.4 (0.2)	Camacho-Muñoz et al. (2010)	1.3	<b>At risk</b>
PRO		Henares-Jarama-Tajo Rivers	0.0073 (0.0021**)	Fernández et al. (2010)	0.3	No risk
PRO		Ebro River	0.0183 (0.0062)	López-Serna et al. (2012)	0.6	No risk
PRO + CAF	0.001; 3.18					
PRO + CAF		Guadiamar River	2.05 + 0.0083	Garrido et al. (2016)	2050 + 0.3	<b>At risk</b>
PRO + CAF		Guadiamar River	2.72 + 0.64	Camacho-Muñoz et al. (2010)	2720 + 2.0	<b>At risk</b>
PRO + CAF		Guadiamar River	2.5 + 0.4	Camacho-Muñoz et al. (2010)	2500 + 1.3	<b>At risk</b>
PRO + CAF		Henares-Jarama-Tajo Rivers	0.41 + 0.0183	Fernández et al. (2010)	410 + 0.6	<b>At risk</b>

**Table 3b**

Risk quotients (RQ) computed as ratio of the measured environmental concentration (MEC) in  $\mu\text{g L}^{-1}$  values of caffeine (CAF) propranolol (PRO) and the mixture of the two compounds to the respective PNEC values in  $\mu\text{g L}^{-1}$  following Approach 2. Mean MECs are indicated in brackets. Surface water bodies at risk are indicated in bold in the column "Risk". \*: mean computed from min and max values. \*\*: median.

PPCP	PNEC	Surface water body	MEC	Reference	RQ	Risk
CAF	0.1	Guadiamar River	2.05 (0.025)	Garrido et al. (2016)	20.5	<b>At risk</b>
CAF		Guadalquivir River	0.23 (0.092)	Robles-Molina et al. (2014)	2.3	<b>At risk</b>
CAF		Guadalquivir River	0.74 (0.39*)	Fernández-Gómez et al. (2013)	7.4	<b>At risk</b>
CAF		Guadiamar River	2.72 (0.68)	Camacho-Muñoz et al. (2010)	27.2	<b>At risk</b>
CAF		Guadiamar River	2.50 (1.37)	Camacho-Muñoz et al. (2010)	25	<b>At risk</b>
CAF		Jarama River	2.14 (1.57)	Valcárcel et al. (2011)	21.4	<b>At risk</b>
CAF		Manzanares River	13.17 (12.92)	Valcárcel et al. (2011)	131.7	<b>At risk</b>
CAF		Guadarrama River	4.72 (2.84)	Valcárcel et al. (2011)	47.2	<b>At risk</b>
CAF		Heneses River	0.67	Valcárcel et al. (2011)	6.7	<b>At risk</b>
CAF		Tagus River	2.08	Valcárcel et al. (2011)	20.8	<b>At risk</b>
CAF		Guadiamar River	1.10 (0.63)	Camacho-Muñoz et al. (2010)	11	<b>At risk</b>
CAF		Henares-Jarama-Tajo Rivers	0.41(0.059**)	Fernández et al. (2010)	4.1	<b>At risk</b>
PRO						
PRO	147	Guadiamar River	0.0083	Garrido et al. (2016)	«0.1	No risk
PRO		Guadiamar River	0.64 (0.26)	Camacho-Muñoz et al. (2010)	«0.1	No risk
PRO		Guadiamar River	0.4 (0.2)	Camacho-Muñoz et al. (2010)	«0.1	No risk
PRO		Henares-Jarama-Tajo Rivers	0.0073 (0.0021**)	Fernández et al. (2010)	«0.1	No risk
PRO		Ebro River	0.0183 (0.0062)	López-Serna et al. (2012)	«0.1	No risk
PRO + CAF	0.1; 147					
PRO + CAF		Guadiamar River	2.05 + 0.0083	Garrido et al. (2016)	20.5 + «0.1	<b>At risk</b>
PRO + CAF		Guadiamar River	2.72 + 0.64	Camacho-Muñoz et al. (2010)	27.2 + «0.1	<b>At risk</b>
PRO + CAF		Guadiamar River	2.5 + 0.4	Camacho-Muñoz et al. (2010)	25 + «0.1	<b>At risk</b>
PRO + CAF		Henares-Jarama-Tajo Rivers	0.41 + 0.0183	Fernández et al. (2010)	4.1 + «0.1	<b>At risk</b>

PNEC is regarded as the concentration below which an unacceptable effect will most likely not occur on surface water taxa (Pennington, 2003). In this study, PNEC values were computed following two different approaches for comparative purposes. According to EC (2003, 2011) two main approaches are possible to determine the predicted no-effect concentration, the deterministic and probabilistic methods. Essentially, the deterministic approach applies an assessment factor to account for uncertainty, while the probabilistic method adopts species sensitivity distribution (SSD) modelling in which all reliable toxicity data are ranked and a model fitted. Where there are insufficient data for a probabilistic

approach, a deterministic approach is adopted, however, where there are sufficient data, both the deterministic and probabilistic approaches will normally be performed (EC, 2011).

In Approach 1 (deterministic), PNEC values were calculated according to the European Union Technical Guidance Document (EC, 2003), that is dividing the lowest sensitivity data, obtained from acute or chronic toxicity studies, available in the literature for several species representing different trophic levels (normally algae, bacteria, invertebrate – *Daphnia* is preferred – and fish species), by an assessment factor (AF). No observed effect concentration (NOEC) was used for chronic toxicity (EC, 2003). For acute

toxicity, the lethal concentration (LC50) or the effect concentration (EC50), that refer to the concentrations at which 50% of their maximal effect (mortality or other effects) is observed in test species, were applied (EC, 2003).

The PNEC values were computed as following:

$$PNEC = \frac{LC50(\text{or } EC50)}{AF} \quad (5)$$

or

$$PNEC = \frac{NOEC}{AF} \quad (6)$$

where AF is the assessment factor that reflects the uncertainty in extrapolating the PNEC values from laboratory toxicity test data from different laboratories. According to EC (2011), the AF may vary from 1 to 1000. Following the EC (2003) an AF of 1000 is to be applied if at least one short-term L(E)C50 from each of the three evaluated trophic levels (fish, invertebrates and algae) is available. An AF of 100 is to be applied to the lowest of two long-term NOECs covering two trophic levels, that is when one long-term NOEC value is available for algae, and crustaceans or fish. An AF of 50 is to be applied when two long-term NOEC values are available for species in two different trophic levels. Finally, an AF of 10 is to be used when NOEC values for species in the three evaluated trophic levels are available (EC, 2003).

LC50 (or EC50) and NOEC values were obtained from this study (LC50 of *D. crassicaudis crassicaudis* and EC50 of *D. magna*) and from the U.S. EPA ECOTOX database ([https://cfpub.epa.gov/ecotox/advanced\\_query.htm](https://cfpub.epa.gov/ecotox/advanced_query.htm)) confining the search to valid data (values preceded by ~, > and < symbols were not accounted as valid). Sensitivity data are showed in the Supplementary File (CAFFEINE; PROPRANOLOL).

In Approach 2 (probabilistic), PNEC values were equal to HC5, i.e. the concentration at which 5% of the species in the SSD exhibit an effect. HC5 was calculated through graphic interpolation on the SSD curves (Maltby et al., 2005). Since SSD should be generated using data for the same type and level of effect, different SSD curves per each compound should have been computed, using either acute (LC50 and EC50) or chronic (NOEC) endpoints from the U.S. EPA ECOTOX database. In this study, we computed the SSD curves considering chronic data only. We did not compute SSD curves based on acute data because no short-term data with algae or other primary producers were available for caffeine from the U.S. EPA ECOTOX database list. SSDs were created using several freshwater taxonomic groups belonging to three ecological categories: primary producers, primary consumers and secondary consumers. At least 8 representative toxicity data for different non-vertebrate species were used according to EFSA (2013). A log-probit distribution was used to model the data and to estimate 95% confidence intervals (Cis), using the spreadsheet provided by the US Environmental Protection Agency (available at: <https://www.epa.gov/caddis-vol4/caddis-volume-4-data-analysis-download-software>, accessed on 01 June 2018).

### 3. Results

#### 3.1. Chemical analyses

Good linearity ( $r^2 > 0.99$ ) was obtained for all target compounds. Values of RSD for the six analytes were below 4% for area and 1% for retention time. The quantification limits ( $S/N = 10$ ) obtained for the compounds were between 0.01 and 0.5 mg L<sup>-1</sup>. As for the stability tests, the concentrations of PPCPs showed no significant changes at

different temperatures and light conditions within four weeks. The effective concentrations measured at the end of the acute tests were within 20% standard deviation from nominal concentrations (CAF: min = 1.4%, max: 3.5%; PRO: min = 0.5%, max = 18.8%).

#### 3.2. Ecotoxicological tests

The results of the tests with the individual compounds (Table 4) showed that both species were much more sensitive to propranolol than to caffeine. Since a remarkably high tolerance to caffeine was assessed, this compound could be considered “practically non-toxic” for both species under acute exposures according to the ecotoxicity categories for aquatic organisms (U.S. EPA, 2012). Previous studies performed with *D. magna* are in agreement with this statement (Supplementary File, CAFFEINE).

Sixty acute toxicity data for caffeine were found in the ECOTOX database and 26 for propranolol, respectively encompassing 7 and 9 taxa (Supplementary File, CAFFEINE, PROPRANOLOL). *D. crassicaudis crassicaudis* was the least sensitive species to acute exposures to caffeine among the freshwater taxa listed in the database (Supplementary File, CAFFEINE). For propranolol, *D. crassicaudis crassicaudis* was more sensitive than *Lemna minor* (standard macrophyte test species) and *Tetrahymena thermophila* (ciliate protist), while it was less sensitive than the remaining 6 taxa in the database (Supplementary File, PROPRANOLOL).

Multiple regression parameters of the mixtures are shown in Table 5 a,b. The parameters were used to calculate the concentrations of mixtures expected to result in 50% of immobilization for each species (Gopalapillai and Hale, 2015), indicating an average sum of toxic units of 0.93 (1.14–0.72) and 0.69 (0.77–0.72) for *D. crassicaudis crassicaudis* and *D. magna*, respectively. Because when  $\sum TU = 1$  CA occurs, these results suggest that the CA model estimated the acute toxicity of the mixture for *D. crassicaudis crassicaudis* better than for *D. magna*. A slightly more than additive deviation from the CA model could explain better the mixture effects of caffeine and propranolol to *D. magna*.

#### 3.3. ERA approach 1

The MEC values of caffeine and propranolol were taken from 7 studies in 8 freshwater bodies in Spain. A total of 12 MECs of caffeine, 5 of propranolol and 4 of the mixture of caffeine and propranolol were considered (Table 3a,b). For both the two compounds, L(E)C50 values were available for crustaceans and fish but not for algae. For caffeine, NOEC data were available for several species belonging to the three trophic levels, being the NOEC of the standard fish species *Salmo salar* the lowest (Supplementary File). An AF equal to 10 was then applied as so to obtain a PNEC = 0.001 µg L<sup>-1</sup>. For propranolol, NOEC data were available for several species belonging to the three trophic levels, being the NOEC of the standard fish species *Danio rerio* the lowest (Supplementary File). An AF equal to 10 was then applied as so to obtain a PNEC = 3.18 µg L<sup>-1</sup>. The RQ values computed for caffeine, propranolol and for the mixture of caffeine and propranolol were all higher than 1 (Table 3a) indicating that the examined freshwater bodies were at risk, except for propranolol in Guadiana River, Henares–Jarama–Tajo catchment and Ebro River.

#### 3.4. ERA approach 2

The SSD curve for caffeine using chronic data was calculated from 97 toxicity data encompassing 10 taxa (Fig. 1) and that for propranolol from 69 toxicity data encompassing 6 taxa (Fig. 2). Both SSD curves were inclusive of at least three trophic levels (Supplementary File; CAFFEINE and PROPRANOLOL). The

**Table 4**

LC50 values at 96 h of *Diacyclops crassicaudis crassicaudis* and EC50 at 48 h of *Daphnia magna* and 95% confidence intervals.

Species	Compound	L(E)C50 ( $\mu\text{g L}^{-1}$ )	CI 95%
<i>Daphnia magna</i>	Caffeine	395000	263000–526000
<i>Daphnia magna</i>	Propranolol	5000	0–12000
<i>Diacyclops crassicaudis crassicaudis</i>	Caffeine	5280000	5248000–5312000
<i>Diacyclops crassicaudis crassicaudis</i>	Propranolol	27000	22000–32000

**Table 5a**

Multiple linear regression analysis of the effect of caffeine and propranolol versus the observed percentage of mortality of *Diacyclops crassicaudis crassicaudis* based on the binary mixture exposures. Parameter estimates (b) for concentrations of caffeine (CAF) and propranolol (PRO) as well as the standardized parameter estimates ( $\beta$ ) are presented.  $R^2 = 0.96$ , Adjusted  $R^2 = 0.95$ .

	Intercept	b	$\beta$	p
	−9.06			0.036*
Caffeine		0.05	1.46	<0.001*
Propranolol		2.16	0.60	<0.001*
CAF x PRO		−0.0017	−0.73	0.055

**Table 5b**

Multiple linear regression analysis of the effect of caffeine and propranolol versus the observed percentage of immobilization of *Daphnia magna* based on the binary mixture exposures. Parameter estimates (b) for concentrations of caffeine (CAF) and propranolol (PRO) as well as the standardized parameter estimates ( $\beta$ ) are presented.  $R^2 = 0.98$ , Adjusted  $R^2 = 0.97$ .

	Intercept	b	$\beta$	p
	−3.92			0.071
Caffeine		0.13	0.84	<0.001*
Propranolol		8.19	0.83	<0.001*
CAF x PRO		0.23	0.22	<0.001*

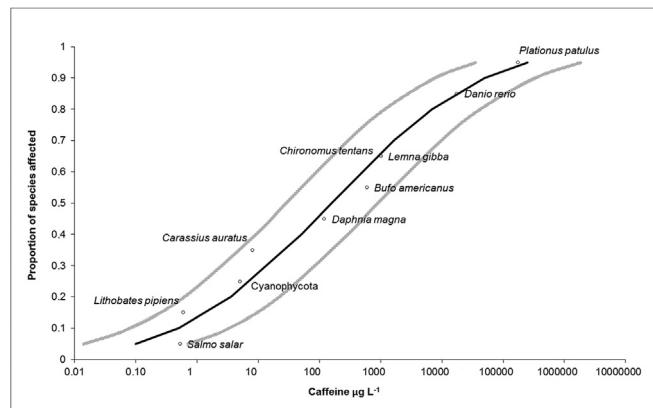


Fig. 1. SSDs of caffeine based on chronic data (Approach 2). The dashed curves shows the 95% confidence intervals.

respective PNEC values calculated from HC5 were  $0.1 \mu\text{g L}^{-1}$  for caffeine and  $147 \mu\text{g L}^{-1}$  for propranolol. No environmental risk was posed by propranolol according to this approach, while caffeine posed a risk in all the investigated freshwater bodies (Table 3b). Finally, the risk posed by the mixtures of propranolol and caffeine in the 4 investigated freshwater bodies was due to caffeine.

#### 4. Discussion

##### 4.1. Sensitivity of *D. crassicaudis crassicaudis* to caffeine and propranolol

Despite that freshwater copepods have been recommended as

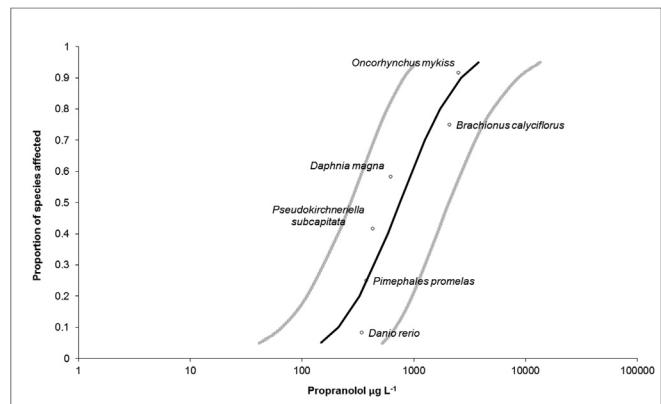


Fig. 2. SSDs of propranolol based on chronic data (Approach 2). The dashed curves shows the 95% confidence intervals.

test organisms in ecotoxicological trials (Kulkarni et al., 2013), the available data are still very few (Di Marzio et al., 2009, 2013, 2018; Di Lorenzo et al., 2014 and references therein) and no studies with freshwater copepods have been performed with caffeine and propranolol before our study. Propranolol showed a significantly higher toxicity to *D. crassicaudis crassicaudis* compared to caffeine. The mechanism of action of this compound in *D. crassicaudis crassicaudis*, and in copepods in general, is completely unknown and has to be investigated in further studies.  $\beta$ -blockers are known to affect aquatic biota, propranolol being the most harmful drug (Fent et al., 2006). This compound can bioaccumulate in aquatic species, as shown by Claessens et al. (2013) and can affect the reproductive outcomes of some crustacean species, such as *D. magna*, reducing the metabolism as a consequence of the depression of heart rate (Dzialowski et al., 2006). However, this effect is not the rule of thumb. The freshwater amphipod *Gammarus* spp. actually doubles its feeding rate and significantly increases its respiration when exposed to propranolol for 7 days, compared to the control (Eriksson Wiklund et al., 2011). The differences in the tested concentrations have likely a role in these conflicting results as also observed by Baldwin and File (1989) who found the anxiolytic effect of high doses of propranolol in rats difficult to interpret. Despite that Jeong et al. (2015) reported inhibitory effects of  $\beta$ -blockers on the heart rate of *D. magna*, along with increased body length and abdominal appendage movements, the presence of  $\beta$ -receptors has never been reported in *D. magna* or other crustaceans (Huggett et al., 2002). Propranolol acute toxicity to *D. magna* is the result of narcosis according to Cleavers (2005), a nonspecific toxicity via disruption of cell membrane integrity. However, an organ-specific effect, vastly different from those of narcotics, has been recently postulated by Jeong et al. (2018).

*D. crassicaudis crassicaudis* was not affected by acute exposures to caffeine if not at concentrations greatly exceeding the compound solubility in water, making it the least sensitive species among those included in the U.S. EPA ECOTOX database. Actually, acute toxicity data with caffeine were well above  $100 \text{ mg L}^{-1}$  for most of the taxa listed in the database. The only clear exception is the

embryo of the frog *Xenopus laevis*, that was highly sensitive to this compound with a mean value of EC50 at 96 h of  $0.19 \pm 0.09 \mu\text{g L}^{-1}$  derived from a vast array of intercalibration trials (Bantle et al., 1994). Nevertheless, the study of DeYoung et al. (1996) returned LC50 values at 5 days ranging from 130 to 190  $\mu\text{g L}^{-1}$  for the same species, indicating a high inter-laboratory variability. Despite that this compound seems to be not lethal to the aquatic biota under acute exposures, if not at very high concentrations, it causes cellular stress in invertebrates through the destabilization of lysosomal membranes (Aguirre-Martinez et al., 2013). While the interactions with adenosine receptors is likely the main mechanism for caffeine's action in mammals (Fredholm et al., 1999), the mechanisms in invertebrates are much less clear. Studies have shown that caffeine interacts with invertebrate ryanodine receptors to release intracellular calcium stores and acts as a phosphodiesterase inhibitor. However its ability to interact with invertebrate adenosine receptors remains an unknown question (Mustard, 2014).

Mixtures may trigger more impairments on aquatic species than the individual mixture components. The mixture toxicity of ibuprofen and diclofenac in the *Daphnia* tests performed by Cleavers (2003), for example, indicated a notable synergistic effect. In our study, an antagonistic effect was somehow expected, since caffeine is a stimulant and propranolol has an opposite effect in humans and rats (Babey et al., 1994; Humayun et al., 1997). However, the effect of propranolol to both *D. crassicaudis* *crassicaudis* and *D. magna* was not weakened by mixing with caffeine. Our results showed that the CA model clearly excluded an antagonistic action of the two compounds for the copepod species. The mixture of the two PPCPs exhibited an even stronger adverse effect in *D. magna* than the individual compounds at the same concentration. Propranolol and caffeine likely did not compete for the same binding site, nor interfere mutually and consequently the suppression of the toxic effect of one PPCP by the other seemed not to occur in the two crustacean species of this study. Similarly, the effect of epinephrine was not blocked by the antagonist propranolol in *D. magna* in the mixture experiments of Postmes et al. (1989) who suggested that the drugs' actions were likely not mediated through adrenoceptors.

#### 4.2. Environmental risk assessment

The occurrence of several PPCPs, including caffeine and propranolol, have been often demonstrated in downstream sites of surface water bodies in the Madrid province, a region with an extensive urban and industrial activity, in the vicinity of the most populated cities, i.e. Madrid, Alcala de Henares and Guadalajara (González-Alonso et al., 2009; Martínez-Bueno et al., 2010). Caffeine and propranolol were detected with high frequency (in more than 80% of the samples) in the Jarama – Tagus system which is the largest drainage basin in the province (Fernandez et al., 2010; Iepure et al., 2017). Fernandez et al. (2010) observed that MECs of some pharmaceutical compounds showed a seasonal variation with a maximum concentration in December (high flow) and a lowest value in September (low flow). The highest MECs of caffeine in the surface water bodies of the Madrid region ranged from 0.001 to  $13.17 \mu\text{g L}^{-1}$  (being the maximum value in the Manzanares River; Fernandez et al., 2010). Propranolol has also been detected in rivers from Barcelona region varying from 0.0004 to  $0.0183 \mu\text{g L}^{-1}$  (being the highest value in the Ebro River; López-Serna et al., 2012). Caffeine seemed to pose an environmental risk to all the investigated freshwater bodies of Spain according to the scenario of Approach 1, both as single compound and in the mixture with propranolol. The latter posed a lower and less frequent risk, according to this scenario. Albeit the PNEC values computed in Approach 2 were slightly different from those in Approach 1, the

relative risk scenario was nevertheless very similar. Propranolol and caffeine MECs considered in this study were in the range of those measured in the municipal wastewater effluents in the United States (propranolol MEC =  $1.9 \mu\text{g L}^{-1}$ ; Huggett et al., 2003), in UK rivers (caffeine MEC up to  $23.778 \mu\text{g L}^{-1}$ ; Ebele et al., 2017), in other European countries (propranolol MECs up to  $0.590 \mu\text{g L}^{-1}$ ; Ternes, 1998) and China (caffeine MECs up to  $8.571 \mu\text{g L}^{-1}$ ; Zhou et al., 2016). Mitigation measures are required whenever the MECs of caffeine exceed  $0.001 \mu\text{g L}^{-1}$ , irrespective of being in a mixture with propranolol or not. Under long-term exposure, even low concentrations of caffeine and propranolol may trigger adverse effects targeting the physiology and behaviour of aquatic species. The effect of chronic exposure to sub-lethal concentrations of caffeine is known to increase activity and alertness, disrupt sleeping patterns and drop learning attitudes and memory in invertebrates (Mustard, 2014). At low concentrations propranolol is known to cause a significant decrease in fecundity and population rates of *D. magna* under chronic exposures (Damasceno de Oliveira et al., 2015).

#### 4.3. Contribution of freshwater copepods to ERA

The RQ values derived from both the two ERA approaches in this study likely underestimate the real risk posed to freshwater ecosystems by caffeine and propranolol since other threats, such as temperature increasing under climate change, may exacerbate biological impairments thus compromising biodiversity and ecosystem functioning. Environmental temperature increasing of  $3^\circ\text{C}$  posed a severe risk for the populations of the freshwater cyclopoid *Eucyclops serrulatus*. The sensitivity to ionized ammonia and to the mixture of ammonia-N and the pesticide Imazamox of both adults and juveniles of this species significantly increased with temperature shift from  $15^\circ\text{C}$  to  $18^\circ\text{C}$  (Di Lorenzo et al., 2015b). On the other hand, the respirometric rates of the groundwater-obligate cyclopoid species *Diacyclops belgicus* did not significantly increase under  $3^\circ\text{C}$  temperature shift from  $14^\circ\text{C}$  to  $17^\circ\text{C}$  (Di Lorenzo and Galassi, 2017a). Further testing is hence recommended since continuous but undetected effects of these pollutants may gradually bioaccumulate, leading to irreversible changes of aquatic communities.

*D. crassicaudis crassicaudis* showed a notable resistance to propranolol, being about 5-fold less sensitive than *D. magna*, and much less sensitive than the other aquatic species of the ECOTOX database to both the two compounds under acute exposures. Intraspecific variability is as a key driver for biodiversity sustenance in ecosystems challenged by environmental change. In this regard, *D. crassicaudis crassicaudis* seems to face propranolol acute contamination events better than other surface water species, thus representing a resistant primary consumer in freshwater ecosystems in adverse conditions.

Pharmaceutical products, as well as other toxicants, might cause a wide variety of adverse effects at sub-lethal concentrations. Oxidative stress was caused by sub-lethal concentrations of propranolol and caffeine in the marine amphipod *Ampelisca brevicornis*, while DNA damage decreased (Maranho et al., 2015). Activation of the protecting antioxidant system (CAT, HSP90 and HSP40 proteins) under short-term exposures to sub-lethal concentrations of ammonia occurs in freshwater cyclopoids (Di Lorenzo et al., 2017), while the expression levels of GSTs and HSP70 enzymes seems to be activated under a variety of toxicants in marine copepods (Lauritano et al., 2012). In future studies, sub-lethal responses related to enzymatic activities, neurotoxicity, oxidative stress and genetic damage should be investigated to assess the possible detrimental effects of caffeine and propranolol to freshwater copepods.

## 5. Conclusions

According to our results, propranolol posed an environmental risk in some but not in all the surface water ecosystems of Spain investigated in this study, while caffeine posed an environmental risk to all the investigated freshwater bodies, both as single compound and in the mixture with propranolol.

In this study we provided the first data about the sensitivity of a freshwater copepod species to caffeine and propranolol. In the individual assays, propranolol was the most toxic compound to *D. crassicaudis crassicaudis*, while caffeine was non-toxic to this species. *D. crassicaudis crassicaudis* showed a notable resistance to propranolol, being about 5-fold less sensitive than *D. magna*, and much less sensitive than other aquatic species to both the two compounds. The toxicity of the propranolol and caffeine was maintained in the mixture and no synergistic or antagonistic effects were observed. The sensitivity of *D. crassicaudis crassicaudis* does not increase the environmental risk posed by the two compounds in the freshwater bodies of Spain, however further testing is recommended since the effect of toxicants on freshwater copepods can occur at sub-lethal concentrations under long term exposures and can be more pronounced under multiple stressors and temperature increasing due to climate change.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2018.12.117>.

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